Journal of Crop Health
https://doi.org/10.1007/s10343-024-00990-0

RESEARCH

Toxicity of Spirotetramat and Cell-free Supernatants of *Xenorhabdus* and *Photorhabdus* Bacteria On the Citrus Mealybug, *Planococcus Citri* and Its Coccinellid Predator, *Cryptolaemus Montrouzieri*

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Received: 7 February 2024 / Accepted: 10 May 2024
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Abstract

Entomopathogenic nematodes (EPNs) are obligate parasites of insects that cooperate with insect-pathogenic bacteria to kill their hosts. Recently, the symbiotic bacteria of EPNs have attracted considerable attention due to their potential use in the control of agricultural pests without their nematode partners. However, the efficacy of these bacteria on the citrus mealybug, *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) remains unknown. In the present study, the separate and joint action of insecticide (spirotetramat) and cell-free supernatants (CFSs) of four bacterial endosymbionts of EPNs (*Xenorhabdus bovienii, X. budapestensis, X. nematophila*, and *Photorhabdus luminescens* subsp. *kayaii*) was evaluated on *P. citri* (nymphs and adults) and its coccinellid predator, *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) (adults) under controlled conditions (25 ± 1 °C, 65 ± 5% RH). The contact application of CFSs of all tested bacteria has resulted in significant mortality in the nymphs and adults of *P. citri* ranging between 80 and 97.5% four days after treatment (DAT) while inducing the same mortality (27.5%) on the adult of *C. montrouzieri*. The combination of the CFSs with full (100 ml/100 l) and half doses of spirotetramat caused similar mortalities in the nymphs and adults of *P. citri* compared to spirotetramat treatments alone. The nymphal stage of *P. citri* was more susceptible to all treatments than the adults. The highest mortality was 52.5 and 100% 4 DAT when the adults and nymphs of *P. citri* were exposed to the CFSs-Spirotetramat combinations, respectively. The results indicate that the CFSs of symbiotic bacteria of EPNs can be as effective as chemical insecticides and enable the reduction of the insecticide application in the control of *P. citri*.

Keywords IPM · Mutualistic bacteria · Biological control · Mealybug · Citrus

Abbreviations

CFSs	Cell-free supernatants
DAT	Days after treatment
EPNs	Entomopathogenic nematodes
RFD	Recommended field dose

Introduction

The citrus mealybug, *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae), is one of the global key pests of citrus orchards and vineyards that cause serious economic losses (Monta et al. 2001; Damavandian 2007; Arshad et al. 2015). The adults and young nymphs of *P. citri* feed on the various plant parts including leaves and fruits which typically results in early fruit and leaf drops as well as fruit deformation and stunted growth (Urbaneja et al. 2020). In addition to direct feeding damage, sticky honeydew produced by *P. citri* serves as a substrate for the growth of sooty-mold fungi which inhibits the photosynthesis of plants by covering the leaves (Demirci et al. 2011; Ülgentürk et al. 2020). In severe infestations, *P. citri* can cause yield reductions of up to 50% in citrus orchards (Kahramanoglu and Usanmaz 2013; Mani 2016). Additionally, damaged fruits generally

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Published online: 15 May 2024

lose their commercial value due to the damaging activities of *P. citri* (Sirisena et al. 2013).

In the last two decades, there has been a dramatic increase in the population density of *P. citri* in the Mediterranean region becoming a pest of major importance to control in citrus orchards and vineyards (Wakgari and Giliomee 2003; Marras et al. 2008; Karacaoğlu and Satar 2017; Mansour et al. 2017). Synthetic insecticides still remain a popular choice for suppressing *P. citri* populations in many parts of the world. However, effective management of *P. citri* populations is challenging due to their tendency to hide in sheltered plant parts, protective wax covering, and small body size (Mansour et al. 2017). Therefore, farmers generally make multiple insecticide applications and/or exceed the maximum application rates during cultivation which generally results in exceeding maximum residue levels (MRLs) (Satar et al. 2012; Garcerá et al. 2013; EFSA 2016; Pigłowski 2022). However, improper and excessive use of insecticides has led to a variety of health and environmental problems including the negative consequences on beneficial insects which may be partly responsible for the recurring outbreaks of *P. citri* (Karacaoğlu and Satar 2017; Rani et al. 2021). In addition, a decrease in the sensitivity of *P. citri* populations to insecticides has also been reported due to extensive applications of insecticides (Kaur and Banu 2019; Swamy et al. 2019). Therefore, it is of utmost importance to incorporate alternative control approaches, such as biological control, into Integrated Pest Management (IPM) programs to minimize the potential risks and impacts of pesticide applications on humans and the environment.

Recently, the bacterial endosymbionts (*Xenorhabdus* and *Photorhabdus* spp.) (Enterobacteriaceae) of entomopathogenic nematodes (EPNs) in the genera *Steinernema* and *Heterorhabditis* (Rhabditida: Steinernematidae and Heterorhabditidae) have stimulated renewed interest in the biological control of pests due to their biocontrol potential against a wide range of insects of agricultural importance (Kepenekci et al. 2015; Canhilal et al. 2017; Özdemir et al. 2021; Askar et al. 2023; Yüksel et al. 2023). In nature, *Xenorhabdus* and *Photorhabdus* spp. bacteria stored in the intestine of infective juveniles (IJs) of EPNs are vectored into the insect haemocoel following the penetration of IJs into the host body and play a key role in host death by releasing a range of immunosuppressant and toxic bioactive compounds into the host hemolymph (Goodrich-Blair and Clarke 2007; Abd-Elgawad 2022; Ogier et al. 2023). The toxemia caused by *Xenorhabdus* and *Photorhabdus* spp. bacteria generally eventuate in the death of host insects within several days (Brown et al. 2006; Subkrasae et al. 2022). Based on fast-killing activity and potent insecticidal properties, recent studies have focused on the biocontrol potential of *in vitro* cultured cell-free supernatants (CFSs) of *Xenorhabdus* and *Photorhabdus* spp. bacterria on various pests and diseases (Chacon-Orozco et al. 2020; Incedayi et al. 2021; Wu et al. 2022). Most of these studies highlighted that CFSs of *Xenorhabdus* and *Photorhabdus* spp. bacteria hold great potential for their insecticidal activity against insects from different orders (Noureldeen et al. 2022; Shaik and Mishra 2023). However, to date, none of these studies have evaluated the efficiency of CFSs of *Xenorhabdus* and *Photorhabdus* spp. bacteria on the *P. citri*. In addition, there is no prior study available reporting the combined efficacy of CFSs of *Xenorhabdus* and *Photorhabdus* spp. bacteria with insecticides. Therefore, this study aimed to explore the insecticidal effects of CFSs of *Xenorhabdus* and *Photorhabdus* spp. bacteria in combination with spirotetramat on different development stages of *P. citri* and its natural enemy, *Cryptolaemus montrouzieri* (Mulsant) (Coccinellidae: Scymninae).

Methods

Galleria Mellonella

The initial population of *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae) larvae was established with the culture obtained from the stock culture of the Entomology Laboratory of Abant Izzet Baysal University (Bolu, Türkiye). The larvae were reared on an artificial diet in 11 glass jars under laboratory conditions. The main constituents of the diet were bran, wheat and corn flour, milk and yeast powder, honey, and glycerin (Metwally et al. 2012). Approximately 100 1st/2nd instar larvae were introduced to each glass jar and maintained at 25 ± 1 °C, 60 ± 5% RH.

Entomopathogenic Nematodes

The last instar larvae of *G. mellonella* were exposed to 100 IJs of EPNs in Petri dishes (9 cm Ø) to obtain freshly emerged IJs. After 48 h of the application, the dead larvae were transferred to Modified White traps to harvest emerged IJs. The harvested IJs were collected in a sterile beaker and rinsed with sterile water. Then, IJs were stored in a Ringer's solution at 9 °C for two weeks (Table 1).

Source of Test Insects

The initial population of *P. citri* culture was established with the nymphs and adults obtained from a stock culture of the Entomology Laboratory of Erciyes University (Kayseri, Türkiye) and reared on the potato sprouts (Muratbey variety). The adults of *C. montrouzieri* (Mulsant) (Coccinellidae: Scymninae) were obtained from Biyotar Company

Table 1 The list of entomopathogenic nematodes and their associated endosymbiotic bacteria used in the bioassays

Nematodes	Accesion Number	Endosymbiotic bacteria	Accesion Number	References
Steinernema feltiae KCS-4S	KX462908	*Xenorhabdus bovienii*	MZ688381	Canhilal et al. (2017) Yüksel et al. (2022)
S. bicornutum MGZ-4S	KX462912	*X. budapestensis*	MW403817	Canhilal et al. (2017) Yüksel et al. (2022)
S. carpocapsae E-76	KX462907	*X. nematophila*	MZ688376	Canhilal et al. (2017) Yüksel et al. (2022)
Heterorhabditis bacteriaphora FLH-4H	KX462939	*Photorhabdus luminescens* subsp. *kayaii*	MZ688382	Canhilal et al. (2017) Yüksel et al. (2022)

(Adana/Türkiye) and the potato sprouts infested by *P. citri* were supplied as food. Both insect cultures were maintained in insect-rearing cages (60 × 60 × 60 cm) under controlled conditions (25 ± 1 °C, 60 ± 5% RH, and a photoperiod of light: dark cycle of 16:8 h).

Isolation of Endosymbiotic Bacteria

The endosymbiotic bacteria used in this study were isolated from four EPN species: *Steinernema feltiae, S. bicornotum, S. carpocapsae* and *Heterorhabditis bacteriophora* (Table 1). The IJs were surface-sterilized in sodium hypochlorite solution (NaClO, 10% w/vol) for 15 min after being washed several times in a Ringer's solution. Then, approximately, surface-sterilized 500 IJs of each EPN species were transferred to Eppendorf tubes (1.5 ml) and crushed thoroughly 1 mL of sterile PBS buffer with an Eppendorf pestle. A 50-µl aliquot of the resulting suspension was streaked onto Petri dishes (9 cm Ø) containing nutrient bromothymol blue triphenyl tetrazolium chloride agar (NBTA medium) (Boemare and Akhurst 1988). Then, Petri dishes were maintained in the dark at 28 °C for 48 h. Pure bacterial colonies were obtained by re-seeding circular primary-form colonies onto the NBTA plates (Bleakley and Nealson 1988). A loop of pure bacterial colony of each strain was injected into *G. mellonella* larvae to confirm the entomopathogenicity of the isolated bacterial strains.

Preparation of Cell-free Supernatants

A single bacterial colony of each isolated bacterial strain was transferred into sterilized Erlenmeyer flasks (250 mL) containing 100 mL Luria-Bertani broth (LB) (Sigma-Aldrich, USA) and grown in a rotary incubator (28 ± 1 °C, 20 ± 5% RH) in full darkness at 150 rpm for one week (Bode 2009; Eroglu et al. 2019). The suspension in the flasks was poured into sterile Falcon tubes (50 mL) and centrifuged at 20,000 rpm for 15 min at 4 °C to separate the bacterial cells. Subsequently, the supernatant solution was passed through a 0.22 µm millipore filter. The filtered supernatants were re-streaked onto the NBTA medium to confirm the final solutions were free of bacterial cells (Boemare and Akhurst 2006). Finally, cell-free supernatant solutions (CFSs) were stored at –20 °C for one week before use.

Toxicity Bioassays

The separate and combined toxicity of spirotetramat (100 g/l, SC, Movento®, Bayer) and CFSs of endosymbiotic bacteria were evaluated on *P. citri* (the 2nd-instar nymphs and adult females) and *C. montrouzieri* (adults). The recommended field dose of spirotetramat (100 ml/100 l) and half of this dose (50 ml/100 l) were applied in combination with the CFSs. The bioassays were conducted in 24-well plates including a filter paper at the bottom of each well. One healthy individual of test insects was transferred into each well with a soft camel hair brush and treated with a 100 µl mixture of CFSs and insecticide. Then, the plates were sealed and maintained at 25 ± 1 °C, 65 ± 5% RH, and a photoperiod of light: dark cycle of 16:8 h. The

Table 2 ANOVA parameters for the mortality of *Planococcus citri* (nymphs and adults) and *Cryptolaemus montrouzieri* (after exposure to cell free supernatants)

Sources	Df	F-Value	*P*-Value
Planococcus citri nymphs			
1st DAT	4	49.692	< 0.001
2nd DAT	4	158.583	< 0.001
3rd DAT	4	179.750	< 0.001
4th DAT	4	173.386	< 0.001
Planococcus citri adults			
1st DAT	4	99.200	< 0.001
2nd DAT	4	62.308	< 0.001
3rd DAT	4	369.450	< 0.001
4th DAT	4	192.643	< 0.001
Cryptolaemus montrouzieri adults			
1st DAT	4	3.964	0.022
2nd DAT	4	21.450	0.001
3rd DAT	4	18.964	0.001
4th DAT	4	30.250	0.001

DAT Day after treatment, *Df* Degree of freedom

Table 3 The mortality (Mean ± SE) of *Planococcus citri* nymphs after 24, 48, 72, 96 h of exposure to cell-free supernatants of symbiotic bacteria

CFSs	Mortality (%) (Mean ± SE)			
	1st DAT	2nd DAT	3rd DAT	4th DAT
Planococcus citri nymphs				
Control (Nutrient Broth)	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa
Xenorhabdus bovienii	37.5 ± 4.7Ba	55.0 ± 2.8Bb	65.0 ± 2.8Bb	90.0 ± 0.0Bc
Xenorhabdus nematophila	50.0 ± 5.7BCa	57.5 ± 2.5Ba	72.5 ± 2.5Bb	82.5 ± 2.5Bc
Xenorhabdus budapestensis	60.0 ± 4.0Ca	77.5 ± 2.5Cb	87.5 ± 2.5Cc	90.0 ± 4.0Bd
Photorhabdus luminescens subsp. *kayaii*	75.0 ± 2.8Da	90.0 ± 4.0Db	90.0 ± 4.0Cb	92.5 ± 4.7Bb
Planococcus citri adults				
Control (Nutrient Broth)	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa
Xenorhabdus bovienii	35.0 ± 2.8Ba	50.0 ± 4.0Bb	60.0 ± 0.0Bc	65.0 ± 2.8Bc
Xenorhabdus nematophila	42.5 ± 2.5Ba	57.5 ± 6.2BCb	92.5 ± 2.5Cc	97.5 ± 2.5Cc
Xenorhabdus budapestensis	65.0 ± 2.8Ca	75.0 ± 2.8BCb	85.0 ± 2.8Cc	87.5 ± 4.7Cc
Photorhabdus luminescens subsp. *kayaii*	55.0 ± 2.8Ca	80.0 ± 4.0Cb	92.5 ± 2.5Cc	97.5 ± 2.5Cc
Cryptolaemus montrouzieri adults				
Control (Nutrient Broth)	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa
Xenorhabdus bovienii	12.5 ± 2.5Ba	17.5 ± 2.5Bab	22.5 ± 2.5Bab	27.5 ± 2.5Bb
Xenorhabdus nematophila	10.0 ± 0.0ABa	17.5 ± 2.5Bb	17.5 ± 2.5Bb	27.5 ± 2.5Bc
Xenorhabdus budapestensis	7.5 ± 2.5ABa	25.0 ± 2.8Bb	25.0 ± 2.8Bb	27.5 ± 2.5Bb
Photorhabdus luminescens subsp. *kayaii*	10.0 ± 4.0ABa	20.0 ± 0.0Bb	25.0 ± 2.8Bb	27.5 ± 2.5Bb

Different capital letters show statistically significant differences among the CFSs of endosymbiotic bacteria for each exposure time (Tukey, $P \leq 0.05$). Different lowercase letters show statistically significant differences among the exposure times for each treatment (Tukey, $P \leq 0.05$)
CFSs Cell-free supernatants of endosymbiotic bacteria of entomopathogenic nematodes, *DAT* Day after treatment, *NB* Nutrient Broth

dead and alive nymphs and adults were recorded daily for four days after treatments. Sterile distilled water and Nutrient Broth prepared in the same proportions were used as control groups and the identical protocol was followed. Each treatment consisted of four replicates, each containing ten adults or nymphs. The bioassays were repeated twice under the same conditions on a different date.

Statistical Analyses

The data was subjected to normality tests using the Shapiro-Wilk (W) test and arcsine transformed. The efficacy of supernatants of endosymbiotic bacteria on *P. citri* and *C. montrouzieri* was analyzed using one-way ANOVA. Multivariate ANOVA (MANOVA) was performed to test the joint toxicity of supernatants of endosymbiotic bacteria and spirotetramat on *P. citri* and *C. Montrouzieri*. Analyses were performed using IBM SPSS statistics version 20.0 for Windows (SPSS Inc., Chicago, IL, USA). Post hoc Tukey's HSD test was used to determine significant differences between groups ($P < 0.05$).

Results

Efficacy of Supernatants

No mortality or symptoms of toxicity were seen in the control treatments of *P. citri* and *C. montrouzieri* during the experiment. However, the application of CFSs of endosymbiotic bacteria resulted in significant mortalities in *P. citri* and *C. montrouzieri* compared to control treatments (Table 2). An increasing trend was observed in *P. citri* and *C. montrouzieri* mortality with increasing exposure time to the CFSs of symbiotic bacteria.

Table 4 Multivariate ANOVA (MANOVA) parameters for the main effects and associated interactions for mortality of *Planococcus* nymphs when exposed to combinations of spirotetramat and cell-free supernatants of endosymbiotic bacteria

Sources	Df	F-value	*P*-value
Dose (D)	1	8.533	0.007
Supernatant (S)	4	5.109	0.003
D*S	4	2.454	0.067
Error-1	30	–	–
Exposure time (t)	3	252.244	< 0.001
t*D	3	6.638	< 0.001
t*S	12	4.390	< 0.001
t*D*S	12	2.839	0.002
Error-2	90	–	–

Df Degree of freedom

Table 5 The mortality (Mean ± SE) of *Planococcus citri* nypmhs after 24, 48, 72, 96 h of exposure to combinations of spirotetramat and cell-free supernatants of endosymbiotic bacteria

Combinations	Mortality (%) (Mean ± SE)			
	1st DAT	2nd DAT	3rd DAT	4th DAT
Control (NB + SDW)	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa
RFD	77.5 ± 2.5Ca	87.5 ± 2.5Cb	95.0 ± 2.8Bb	100.0 ± 0.0Bb
RFD + X. bov	75.0 ± 2.8Ca	80.0 ± 0.0BCa	85.0 ± 2.5Ba	95.0 ± 2.8Bb
RFD + X. nem	70.0 ± 4.0Ca	77.5 ± 2.7BCa	87.5 ± 4.7Bb	92.5 ± 4.7Bb
RFD + X. buda	72.5 ± 4.7Ca	75.0 ± 4.8BCa	90.0 ± 5.7Bb	92.5 ± 4.7Bb
RFD + P.l.	57.5 ± 4.7Ba	67.5 ± 2.5Ba	82.5 ± 4.7Bb	95.0 ± 2.8Bb
Control (NB + SDW)	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa
RFD/2	50.0 ± 4.0Ba	67.5 ± 2.5Ba	92.5 ± 4.7Cb	97.5 ± 2.5Bb
RFD/2 + X. bov	72.5 ± 2.5Ca	77.5 ± 2.5Ca	85.0 ± 2.8BCa	100.0 ± 0.Bb
RFD/2 + X. nem	55.0 ± 2.8Ba	62.5 ± 2.5Ba	77.5 ± 2.5Bb	92.5 ± 4.7Bc
RFD/2 + X. buda	75.0 ± 2.8Ca	77.5 ± 2.5Ca	85.0 ± 5.0BCab	95.0 ± 5.0Bb
RFD/2 + P.l.	55.0 ± 2.8Ba	65.0 ± 2.8Ba	77.5 ± 2.5Bb	92.5 ± 4.7Bc

Different capital letters show statistically significant differences among the exposure times for each treatment. Different lowercase letters show statistically significant differences among the exposure times for each treatment (Tukey, $P \leq 0.05$)
RFD Recommended field dose, *X. bov. Xenorhabdus bovienii*, *X. nem. Xenorhabdus nematophila*, *X. buda. Xenorhabdus budapestensis*, *P.l. Photorhabdus luminescens* subsp. *kayaii*, *DAT* Day after treatment, *NB* Nutrient Broth, *SDW* Sterile distilled water

The CFSs of *X. bovienii* showed relatively poor efficacy on *P. citri* nymphs and adults compared to other symbiotic bacteria tested except for the mortality (90%) on the nymphs of *P. citri* at the 4th day after treatment (DAT). However, after 4 days of exposure to CFSs, *P. luminescens* subsp. *kayaii* was the only strain that achieved mortality of over 90% on the *P. citri* nymphs and adults. Only two CFSs of symbiotic bacteria (*P. luminescens* subsp. *kayaii* and *X. budapestensis*) induced a mortality of over 50% on the adults of *P. citri* at the 1st DAT. The CFSs of *X. budapestensis* caused the highest mortality (65%) on *P. citri* adults at the 1st DAT (Table 3). The CFSs applied alone caused very low mortality on *C. montrouzieri* adults and the mortality did not exceed 27.5% after 4 days of exposure to CFSs (Table 3).

Combined Efficacy of Spirotetramat and Supernatants

All main factors (dose, supernatant, and exposure time) significantly affected the overall mortality of *P. citri* nymphs (Table 4). The results indicated that the nymphs of *P. citri* were more susceptible to sipirotetramat and CFSs than the adults. Recommended field doses (RFD) of spirotetramat alone caused higher mortality to the nymphs of *P. citri* than its combinations with CFSs of symbiotic bacteria during the experiment and maximum nymphal mortality (100%) was obtained at the 4th DAT. The RFD + P.l. combination showed poor efficacy at the 1st and 2nd DAT compared to other combinations tested. However, all CFS combinations induced mortality of over 90% at the 4_{th} DAT. The half-dose combination of spirotetramat (RFD/2) with CFSs *X. bovienii* and *X. budapestensis* notably increased the nymphal mortality at the 1st and 2nd DAT compared to other treatments. The maximum nymphal mortality (100%) was achieved by the combinations of RFD/2 with *X. bovienii* at the 4th DAT (Table 5).

All main variables influenced the mortality of adults except for the CFSs of symbiotic bacteria (Table 6). The adults of *P. citri* showed a lower susceptibility to combinations of RFD of spirotetramat with CFSs and the mortality did not exceed 52.5% after four days of exposure. The combinations of RFD of spirotetramat with CFSs exhibited greater mortality than RFD of spirotetramat treatment alone. The RFD of spirotetramat and *X. nematophila* combination yielded the highest mortality (40%) at the 1st DAT. However, the mortality was significantly lower when the adults were exposed to the half-dose combina-

Table 6 Multivariate ANOVA (MANOVA) parameters for the main effects and associated interactions for mortality levels of *Planococcus citri* adults when exposed to combinations of spirotetramat and cell-free supernatants of endosymbiotic bacteria

Sources	Df	F-value	*P*-value
Dose (D)	1	72.200	< 0.001
Supernatant (S)	4	2.300	0.082
D*S	4	4.061	0.010
Error-1	30	–	–
Exposure time (t)	3	137.014	< 0.001
t*D	3	6.288	< 0.001
t*S	12	1.397	0.182
t*D*S	12	1.305	0.230
Error-2	90	–	–

Df Degree of freedom

Table 7 The mortality (Mean ± SE) of *Planococcus citri* adults after 24, 48, 72, 96 h of exposure to combinations of spirotetramat and cell-free supernatants of endosymbiotic bacteria

Combinations	Mortality (%) (Mean ± SE)			
	1st DAT	2nd DAT	3rd DAT	4th DAT
Control (NB + SDW)	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa
RFD	20.0 ± 4.0Ba	27.5 ± 4.7Bab	30.0 ± 4.0Bab	45.0 ± 5.0Bb
RFD + X. bov	22.5 ± 4.7BCa	30.0 ± 4.0Bab	40.0 ± 4.0BCb	47.5 ± 2.5Bb
RFD + X. nem	40.0 ± 4.0Ca	40.0 ± 4.0Ba	50.0 ± 4.0Cb	52.5 ± 4.7Bb
RFD + X. buda	20.0 ± 4.0Ba	25.0 ± 2.8Ba	35.0 ± 2.8BCb	47.5 ± 4.7Bb
RFD + P.l.	30.0 ± 4.0BCa	35.0 ± 2.8Ba	42.5 ± 2.5BCab	52.5 ± 2.5Bb
Control (NB + SDW)	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa
RFD/2	17.5 ± 2.5Ba	20.0 ± 0.0Ba	30.0 ± 0.0Bb	32.5 ± 2.5Bb
RFD/2 + X. bov	15.0 ± 2.8Ba	22.5 ± 2.5Bab	25.0 ± 2.8Bab	35.0 ± 2.8Bb
RFD/2 + X. nem	15.0 ± 2.8Ba	22.5 ± 2.5Ba	25.0 ± 2.8Ba	27.5 ± 2.5Ba
RFD/2 + X. buda	10.0 ± 0.0Ba	22.5 ± 2.5Bab	22.5 ± 2.5Bab	30.0 ± 4.0Bb
RFD/2 + P.l.	10.0 ± 0.0Ba	17.5 ± 2.5Bb	22.5 ± 2.5Bb	25.0 ± 2.8Bb

Different capital letters show statistically significant differences among the exposure times for each treatment. Different lowercase letters show statistically significant differences among the exposure times for each treatment (Tukey, $P \leq 0.05$)
RFD Recommended field dose, *X. bov. Xenorhabdus bovienii*, *X. nem. Xenorhabdus nematophila*, *X. buda. Xenorhabdus budapestensis*, *P.l. Photorhabdus luminescens* subsp. *kayaii*, *DAT* Day after treatment, *NB* Nutrient Broth, *SDW* Sterile distilled water

Table 8 Multivariate ANOVA (MANOVA) parameters for the main effects and associated interactions for mortality levels of *Cryptolaemus montrouzieri* adults when exposed to combinations of spirotetramat and cell-free supernatants of endosymbiotic bacteria

Sources	Df	F-value	*P*-value
Dose (D)	1	12.617	0.001
Supernatant (S)	3	0.652	0.630
D*S	9	0.175	0.949
Error-1	30	–	–
Exposure time (t)	3	107.447	< 0.001
t*D	3	2.085	0.108
t*S	12	0.824	0.625
t*D*S	12	0.676	0.771
Error-2	90	–	–

Df Degree of freedom

tions of spirotetramat (RFD/2) with CFSs. The highest efficacy (35%) was obtained from the combination of RFD/2 of spirotetramat with *X. bovienii* at the 4th DAT although no significant mortality was observed among treatments (Table 7).

The dose of spiretetramat and the exposure time were significant on the mortality rates of *C. montrouzieri* while the effects of CFSs of symbiotic bacteria were found insignificant (Table 8). The alone and combined applications of spirotetramat and CFSs showed low toxicity to *C. montrouzieri* adults and the mortality rates did not exceed 22.5% when the adults were exposed to the combinations of RFD of spirotetramat with CFSs. The susceptibility of *C. montrouzieri* adults was lower in the half-dose combination of spirotetramat (RFD/2) with CFSs than RFD and remained below 10% mortality at the 2nd DAT (Table 9).

Discussion

The current study focused on examining the biocontrol potential of mutualistic bacteria of EPNs alone and in combination with spirotetramat in the control of *P. citri* and its natural enemy, *C. montrouzieri*. The results revealed that contact application of CFSs of *Xenorhabdus* and *Photorhabdus* bacteria were quite lethal for the nymphs and adults of *P. citri* while showing a low toxicity to *C. montrouzieri*. This finding is in line with that of a previous study by Wu et al. (2022) who reported 85% mortality in another hemipteran species, *Monellia caryella* (Fitch) (Hemiptera: Aphididae) after exposure to *P. luminescent* and *X. bovienii*. Similar to our study, Wu et al. (2022) also documented that bacterial metabolites of *P. luminescent* and *X. bovienii* had low toxicity (30%) against non-target arthropod, the lady beetle, *Harmonia axyridis* (Coleoptera: Coccinellidae). The differences in the susceptibility of *P. citri* and *C. montrouzieri* against the CFSs are most likely due to the physical protection of beetle elytra. Beetle elytra has a thicker cuticular structure than mealybugs and might have served as a shield against CFSs (Zhou et al. 2023). In addition, *P. citri* might have been more exposed to CFSs due to crawler dispersal behavior with surfaces treated with CFSs. Similar to our findings, in a previous study, poor efficacy of *Xenorhabdus szentirmaii* and *X. nematophila* supernatants on *Phytoseiulus persimilis* and *Neoseiulus californicus* (Acari: Phytoseiidae) was attributed to ticker cuticular structure of the predatory mites and less contact with surfaces (Cevizci et al. 2020). Moreover, all CFSs treatments induced the same mortality in *C. montrouzieri* adults at 4 DAT, suggesting that the absorption of CFSs by the cuticle of *C. mon-*

Table 9 The mortality (Mean ± SE) of *Cryptolaemus montrouzieri* adults after 24, 48, 72, 96 h of exposure to combinations of spirotetramat and cell-free supernatants of endosymbiotic bacteria

Combinations	Mortality (%) (Mean ± SE)			
	1st DAT	2nd DAT	3rd DAT	4th DAT
Control (NB + SDW)	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa
RFD	5.0 ± 2.8Aa	7.5 ± 2.5Ba	15.0 ± 2.8Bab	22.5 ± 2.5Bb
RFD + X. bov	2.5 ± 2.5Aa	12.5 ± 2.5Bab	17.5 ± 2.5Bab	22.5 ± 2.8Bb
RFD + X. nem	2.5 ± 2.5Aa	5.0 ± 2.8Ba	17.5 ± 2.5Bb	22.5 ± 2.5Bb
RFD + X. buda	2.5 ± 2.5Aa	7.5 ± 2.5Ba	15.0 ± 2.8Bb	22.5 ± 2.5Bb
RFD + P.l.	10.0 ± 0.0Ba	10.0 ± 0.0Ba	15.0 ± 2.8Bab	22.5 ± 2.5Bb
Control (NB + SDW)	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa
RFD/2	2.5 ± 2.5Aa	7.5 ± 2.5Aa	12.5 ± 2.5Bb	17.5 ± 2.5Ba
RFD/2 + X. bov	2.5 ± 2.5Aa	5.0 ± 2.8Aa	12.5 ± 2.5Bb	17.5 ± 4.7Bb
RFD/2 + X. nem	0.0 ± 0.0Aa	7.5 ± 2.5Aab	10.0 ± 0.0Bab	17.5 ± 2.5Bb
RFD/2 + X. buda	0.0 ± 0.0Aa	5.0 ± 2.8Aa	12.5 ± 2.5Bb	15.0 ± 2.8Bb
RFD/2 + P.l.	5.0 ± 2.8Aa	7.5 ± 2.5Aa	12.5 ± 2.5Ba	15.0 ± 2.8Ba

Different capital letters show statistically significant differences among the CFSs of endosymbiotic bacteria for each exposure time. Different lowercase letters show statistically significant differences among the exposure times for each treatment (Tukey, $P \leq 0.05$)
RFD Recommended field dose, *X. bov. Xenorhabdus bovienii*, *X. nem. Xenorhabdus nematophila*, *X. buda. Xenorhabdus budapestensis*, *P.l. Photorhabdus luminescens* subsp. *Kayaii*, *DAT* Day after treatment, *NB* Nutrient Broth, *SDW* Sterile distilled water

trouzieri might have played a more critical role in mortality than bacterial metabolites. However, in the case of *P. citri*, the results showed that the CFSs of *Xenorhabdus* and *Photorhabdus* bacteria were highly toxic causing mortalities ranging between 65.0–97.5%. The difference in the insecticidal activities of CFSs might be related to toxin complexes and amount of secondary metabolites (Cimen et al. 2022). Hasan et al. (2019) reported that the synthesis of secondary metabolites differed among *X. nematophila* strains and was positively correlated with insect mortality. *Xenorhabdus* and *Photorhabdus* bacterial strains produce a high diversity of metabolites with different insecticidal and immunosuppressant bioactivities (Dreyer et al. 2018; Wenski et al. 2020). The composition and amounts of bioactive compounds play a crucial role in the mortality of host insects (Dowling and Waterfield 2007; Vicente-Díez et al. 2021).

In the present study, the efficacy of spirotetramat alone and in combination with CFSs were also evaluated. Spirotetramat is one of the widely used insecticides in the control *P. citri* with a mode of action that acts as an inhibitor of lipid biosynthesis (IRAC 2012). Due to its broad-spectrum activity against sucking insects, spirotetramat is heavily used on various crops including citrus orchards and vineyards (Maus 2008; Afzal et al. 2018; Ghafoor et al. 2019). Although spirotetramat was classified as low toxic to non-target organisms, several studies have documented its adverse effects on predators and parasitoids (Franco et al. 2004; Fernández et al. 2015; Francesena et al. 2017; Zeinadini et al. 2019). However, in the present study, the mortality of *C. montrouzieri* adults did not exceed 22.5% when exposed to RFD of spirotetramat. These results are quite similar to the findings of Planes et al. (2013) who reported 20% mortality in *C. montrouzieri* adults after direct spirotetramat application. On the other hand, RFD of spirotetramat was highly lethal for the nymphs of *P. citri* while exhibiting moderate lethality in the adults. Similar to our studies, Satar et al. (2013) reported 100% mortality in *P. citri* nymphs after 7 days of exposure to spirotetramat. Previous studies also documented lower mortality in the adults of *P. citri* and other species exposed to spirotetramat compared to the nymphal stages (Franco et al. 2004; Assouguem et al. 2021). Since no studies have reported spirotetramat resistance for *P. citri* to date, the lower susceptibility of *P. citri* adults to spirotetramat is most probably associated with the waxy coating over their bodies that shields them from insecticides (Venkatesan et al. 2016; Ulusoy et al. 2022).

Conclusion

To our knowledge, this is the firts study evaluating joint action of insecticide (spirotetramat) and cell-free supernatants of endosymbiotic bacteria bacteria of EPNs on *P. citri* and its coccinellid predator, *C. montrouzieri*. In the present study, similar mortalities were achieved by combining spirotetramat with CFSs compared to spirotetramat applications alone, indicating that CFSs of endosymbiotic bacteria are compatible with spirotetramat and can help reduce the amount of insecticides released into the environment. The CFSs of *Xenorhabdus* and *Photorhabdus* bacteria have great potential to be included in Integrated Pest Management (IPM) programs and can contribute to the eco-friendly management of *P. citri*.

Supplementary Information The online version of this article (https://doi.org/10.1007/s10343-024-00990-0) contains supplementary material, which is available to authorized users.

Acknowledgements This study is a part of Merve Özdemir's Master's thesis at the Graduate School of Natural and Applied Sciences of Erciyes University. The authors would like to thank Prof. Dr. Ramazan CANHİLAL, Prof. Dr. Murat MUŞTU, and Dr. Lerzan ERKILIÇ for their assistance in obtaining test insects and nematodes.

Author Contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by M. Özdemir. The first draft of the manuscript was written by E. Yüksel and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Availability of data and materials The data and materials are available on request from the corresponding author.

Conflict of interest M. Özdemir and E. Yüksel declare that they have no competing interests.

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